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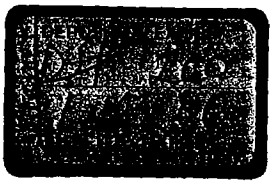
(57) Abstract

The present invention relates to new oligonucleotide arrays and methods of using oligonucleotide arrays. Binary oligonucleotide arrays, having binary oligonucleotides characterized by a constant nucleotide sequence adjacent to a variable nucleotide sequence, are used for sorting and surveying nucleic acid strands. Oligonucleotide arrays are used for sorting mixtures of nucleic acid strands, making immobilized partial copies of nucleic strands, ligating strands, or introducing site directed mutations into strands. Information is obtained for determining the sequence of a nucleic acid strand, alone or in a mixture, by generating partials of the strand and, for groups of partials having the same terminal variable oligonucleotide, separately determining the presence and sequence of all variable oligonucleotides. Arrays are also used to order previously sequenced nucleic acid fragments and to allocate ordered allelic fragments to chromosomal linkage groups.



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NOVEL OLIGONUCLEOTIDE ARRAYS AND THEIR USE FOR SORTING, ISOLATING, SEQUENCING, AND MANIPULATING NUCLEIC ACIDS

Field of the Invention

This invention is in the field of sorting, isolating, sequencing, and manipulating nucleic acids.

Background of the Invention

Ordered arrays of oligonucleotides ("oligos") immobilized on a solid support have been proposed for sequencing DNA fragments. It has been recognized that hybridization of a cloned single-stranded DNA fragment to all possible oligo probes of a given length can identify the corresponding, complementary oligo segments that are present somewhere in the fragment, and that this information can sometimes be used to determine the DNA sequence. Use of arrays can greatly facilitate the surveying of a DNA fragment's oligo segments.

In an oligonucleotide array each oligo probe is immobilized on a solid support at a different predetermined position. The array allows one to simultaneously survey all the oligo segments in a DNA fragment strand. Many copies of the strand are required, of course. Ideally, surveying is carried out under conditions to ensure that only perfectly matched hybrids will form. Oligo segments present in the strand can be identified by determining those positions in the array where hybridization occurs. The nucleotide sequence of the DNA sometimes can be ascertained by ordering the identified oligo segments in an overlapping fashion. For every identified oligo segment, there must be another oligo segment whose sequence overlaps it by all but one nucleotide. The entire sequence of the DNA strand can be represented by a series of overlapping oligos, each of equal length, and each located one nucleotide further along the sequence. As long as every overlap is unique, all of the identified oligos can be assembled into a contiguous sequence block.

There is an important limitation to sequencing by known surveying techniques. As relatively longer DNA strands are surveyed, there is an increasing probability that more than two

identified oligos will share the same overlapping sequence, i.e., the overlap is not unique. When this occurs, the sequence of the DNA cannot be unambiguously determined. Instead of one contiguous sequence block that contains the entire DNA sequence, the oligos can only be assembled into a number of smaller sequence blocks, whose order is not known.

Summary of the Invention

We have invented new oligonucleotide arrays and methods of using them.

A "binary array" according to the invention contains immobilized oligos comprised of two sequence segments of predetermined length, one variable and the other constant. The constant segment is the same in every oligo of the array. The variable segments can vary both in sequence and length. Binary arrays have advantages compared with ordinary arrays: (1) they can be used to sort strands according to their terminal sequences, so that each strand binds to a fixed location (an address) within the array; (2) longer oligos can be used on an array of a given size, thereby increasing the selectivity of hybridization; this allows strands to be sorted according to the identity of internal oligo segments adjacent to a particular constant sequence (such as a segment adjacent to a recognition site for a particular restriction endonuclease), and this allows strands to be surveyed for the presence of signature oligos that contain a constant segment in addition to a variable segment; (3) universal sequences, such as priming sites, can be introduced into the termini of sorted strands using the binary arrays, thereby enabling the strands' specific amplification without synthesizing primers specific for each strand, and without knowledge of each strand's terminal sequences; and (4) the specificity of hybridization during surveying can be increased by coupling hybridization to a ligation event that discriminates against terminal basepair mismatches.

A "sectioned array" as used herein is one divided into sections, so that every individual area is mechanically separated

from all other areas, such as, for example, a depression on the surface, or a "well". The areas have different oligos immobilized thereon. A sectioned array allows many reactions to be performed simultaneously, both on the surface of the solid support and in solution, without mixing the products of different reactions. The reactions occurring in different wells are highly specific due to the nucleotide sequence of the immobilized oligo. A large number of sortings and manipulations of nucleic acids can be carried out in parallel, by amplifying or modifying only those nucleic acids in each well that are perfectly hybridized to the immobilized oligos. Nucleic acids prepared on a sectioned array can be transferred to other arrays (replicated) by direct blotting of the wells' contents (printing), without mixing the contents of different wells of the same array. Furthermore, the presence of individual sections in arrays allows multiple rehybridizations of bound nucleic acids to be performed, resulting in a significant increase in hybridization specificity. It is particularly advantageous according to this invention to use a binary array that is sectioned.

Our invention includes methods of using sectioned arrays to sort mixtures of nucleic acid strands, either RNA or DNA. As used herein, "strand" means not just a single strand, but multiple copies thereof; and "mixture of strands" means a mixture of copies of different strands no matter how many copies of each are present. Similarly "fragment" refers to multiple copies thereof, and "mixture of fragments" means a mixture of copies of different fragments. The methods include sorting strands either according to their terminal oligo segments (3'-terminal or 5'-terminal), or according to their internal oligo segments on a binary array. Before or after sorting, universal priming region(s) can be added to the strands' termini to enable amplification. Binary sectioned arrays for sorting according to strands' terminal sequences ("terminal sequence sorting arrays") can be comprehensive. A "comprehensive array" is one wherein any possible strand will hybridize to at least one immobilized oligo. This type of sorting is particularly useful for preparing comprehensive libraries of fragments of a large genome. For example, in one

embodiment of the invention, strands of restriction fragments have their restriction sites restored and are sorted on a binary array. That array contains immobilized oligos whose constant segments contain the sequence complementary to the restriction site, and an adjacent variable segment. The array is complete, containing all variable sequences of each type in separate areas.

Our invention also includes using sectioned arrays for preparing every possible partial copy of a strand or a group of strands. The term "partial" refers to multiple copies thereof. Partials are prepared by either of the following methods: (1) terminal sorting on a binary sectioned array of a mixture of all possible partial strands generated by random degradation of a parental strand; or (2) generation of partials directly on an array, through the sorting on an ordinary sectioned array of parental strands according to the identity of their internal oligo sequences, followed by the synthesis of partial copies of each parental strand by enzymatic extension of the immobilized oligos utilizing the hybridized parental strands as templates. In either case, generated partials correspond to a parental strand whose 3' or 5' end is truncated to all possible extents (at the "variable" end of the partial), and whose other end is preserved (at the "fixed" end of the partial). These are "one-sided partials." Unless otherwise indicated the word "partial" is used herein to refer to one-sided partials.

Our invention also includes methods of using oligo arrays to obtain oligo information as part of a process for determining the nucleotide sequence of a long nucleic acid strand, or of many nucleic acid strands in an unknown mixture. A complete set of one-sided partials of the strand or strands is prepared on a sectioned array, and the oligo content of the partial strands in each well of the array is separately surveyed (i.e. each group of partials sharing the same oligo at the partials' variable end is surveyed).

Our invention also includes methods of using oligo arrays for ordering previously sequenced fragments from a first restriction digest of a large nucleic acid or even a genome.

Our invention also includes methods of using oligo arrays for allocating sequenced and ordered allelic fragments into their chromosomal linkage groups.

Our invention also includes a method of using binary arrays for surveying the oligos contained in strands or their partials. This method provides improved comprehensive surveys over the conventional surveying of oligos on an ordinary array.

Brief Description of the Drawings

Figure 1 shows a binary array.

Figure 1a shows an oligo immobilized in an area of a binary array.

Figure 2 shows a sectioned array having depressions.

Figure 2a shows a well of a sectioned array.

Figure 3 shows addition of a lattice to a support to make a sectioned array.

Figure 4 shows an example of sorting and amplification of restriction fragments on a sectioned binary array.

Figure 5 shows an example of preparing partials on a sectioned ordinary array.

Figure 6 shows, schematically, the order of steps for sequencing a complete genome.

Figure 7 shows, schematically, the use of a sheet with a number of miniature survey arrays for simultaneous surveying every well in a partialing array.

Figures 8 to 11 show examples of the determination of nucleotide sequences from indexed address sets obtained from analysis of mixtures of strands.

Detailed Description of the Invention

I. Oligonucleotide arrays

As used herein an "oligonucleotide array" is an array of regularly situated areas on a solid support wherein different oligos are immobilized, typically by covalent linkage. Each area contains a different oligo whose location is predetermined.

Arrays can be classified by the composition of their immobilized oligos. "Ordinary arrays" contain oligos comprised entirely of "variable segments". Every position of the oligo sequence in such a segment can be occupied by any one of the four commonly occurring nucleotides.

Comprehensive ordinary arrays are those wherein any segment of any possible strand will hybridize perfectly to the length of one or more immobilized oligos so that no strand is lost.

Binary arrays differ from ordinary arrays. A binary array is illustrated in Figures 1 and 1a. Figure 1 shows a substrate or support 1 having immobilized thereon an array of oligos 3, each oligo being in a separate area 2 of support 1. Figure 1a shows one area 2. A binary oligo 3 (many copies, of course) comprised of constant region 5 and variable region 6 is covalently bound to support 1 by covalent linking moiety 4.

Because of the constant segments, binary arrays provide means for the hybridization of longer sequences without increasing the size of the array. The constant segment can be located within the immobilized oligo either "upstream" of the variable segment (i.e., toward or at the 5' end of the oligo) or "downstream" from the variable segment (i.e., toward or at the 3' end of the oligo). The type of array that is chosen depends on the specific application. The constant region preferably is or includes a good priming region for amplification of hybridized strands by a polymerase chain reaction (PCR), or a promoter for copying the strand by transcription. Generally a length of 15 to 25 nucleotides is suitable for priming. The constant region can contain all or part of the complement of a restriction site. A binary array can be "plain" or "sectioned" (see below).

"Plain arrays" known in the art are arrays in which the individual areas are not physically separated from one another. Reactions carried out simultaneously are limited to those in which the nucleic acid templates and the reaction products are bound in some manner to the surface of the array to avoid the intermixing of products.

"Sectioned arrays" are divided into sections, so that each area is physically separated by mechanical or other means (e.g.,

a gel) from all the other areas, e.g., depressions on the surface, called a "well". There are many techniques apparent to one skilled in the art for preventing the exchange of materials between areas; any such method can be used to make a "sectioned" array, as that term is used herein, even though there might not be a physical wall between areas.

One type of sectioned array is illustrated in Figures 2 and 2a. Figure 2 shows a support sheet 60 having an array of depressions or wells 62, each containing many copies of an immobilized oligo 64. Figure 2a shows one well 62 of the array of Figure 2. Well 62 formed in support 60 has therein oligo 64 covalently bound to support 60 by covalent linking moiety 66. In practice one may prepare a plain array, e.g., on a flat sheet, and then, at a point during a series of steps involving its use, convert the array into a sectioned array, e.g., by making physical depressions in a deformable solid support to isolate the individual areas. The sectioned array can also be created by applying a lattice to the solid support and bonding it to the surface so that each area is surrounded by impermeable walls. An exploded perspective view of such a sectioned array is shown in Figure 3. Support or substrate 70, here a planar sheet, has mounted thereon and affixed thereto a lattice 72 comprised of a series of horizontal members 74, 76. The lattice members define a series of open areas which, in conjunction with support 70, define an array of wells 78. In some applications it is preferable to utilize a detachable lattice (or a removable cover sheet), so that the sectioned array can be converted back to a plain array.

Sectioned arrays according to this invention can be used to increase the specificity of hybridization of nucleic acids to the immobilized oligos. After hybridization, unhybridized strands can be washed away. Hybridized strands can then be released into solution without mixing. Released strands can be rebound to the immobilized oligos, and unhybridized strands can be washed away. Each successive release, rebinding, and washing increases the ratio of perfectly matched hybrids to mismatched hybrids.

An array can be "3'" or "5'". "3' arrays" possess free 3' termini and "5' arrays" possess free 5' termini. The immobilized oligos in a 3' array can be extended at their 3' termini by incubation with a nucleic acid polymerase. If it is a template-directed polymerase, only immobilized oligos hybridized to a template strand can be extended.

Methods of oligodeoxyribonucleotide synthesis directly on a solid support are also known in the art, including methods wherein synthesis occurs in the 3' to 5' direction (so that the oligos will possess free 5' termini). Methods wherein synthesis occurs in the 5' to 3' direction (so that the oligos will possess free 3' termini) are also known.

Suitable substrates or supports for arrays should be non-reactive with reagents to be used in processing, washable under stringent conditions, not interfere with hybridization and not be subject to inordinate non-specific binding. For example, treated glass polymers of various kinds (e.g., polyamide and polyacromorpholide), latex-coated substrates and silica chips.

Arrays can be made over a wide range of sizes. In the example of a square sheet, the length of a side can vary from a few millimeters to several meters.

II. Sorting nucleic acids

Our invention allows mixtures of strands to be sorted according either to their terminal oligo segments ("terminal sorting") or their internal oligo segments ("internal sorting") on a binary array.

There are two important aspects of our invention for sorting. First, each strand in a mixture can be made to hybridize at only a few, or a single, location. And second, each strand can be provided with universal terminal priming regions that enable PCR amplication without prior knowledge of the terminal nucleotide sequences and without the need to synthesize individual primers.

For terminal sorting, the priming region(s) can be made essentially dissimilar from the sequences occurring in the

nucleic acids that are present in the mixture to be sorted, so that priming does not occur anywhere but at the strands' termini. When strands from a complete restriction digest of a DNA are to be terminally sorted and amplified, priming only at the strands termini can be promoted by restoring the terminal restriction sites (those sites having been eliminated from internal regions by complete digestion) concomitant with the generation of terminal priming regions.

Terminal sorting is carried out on a binary array, which preferably is sectioned. The immobilized oligos contain a constant segment complementary to either the strands' 3' priming region or 5' priming region. Thus, each strand can only be hybridized to one location within the array. By sorting on a comprehensive array, every strand is bound somewhere within the array. This is especially important for the preparation of a comprehensive library of fragments of a long nucleic acid or a genome.

Strands can be sorted on either 3' or 5' arrays in which the constant segment is located either upstream or downstream of the variable segment. High specificity of sorting can be achieved by employing 3' arrays in which the constant segment of the immobilized oligos is upstream. In that case, sorting can be followed by the generation of an immobilized copy of each sorted strand using the immobilized oligos as primers for the synthesis of a complementary copy of that strand when the array is incubated with an appropriate DNA polymerase. The generation of copies covalently linked to the array enables the array to be vigorously washed to remove non-covalently bound material before strand amplification. It also enables the arrays to serve as permanent banks of sorted strands which can subsequently be amplified over and over to generate copies for further use.

A strand sorting procedure is shown in Figure 4. A DNA sample 10 is completely digested with a restriction endonuclease. The ends of each fragment are restored, and universal priming sequences 17 generated in the process to prepare fragments 11 for sorting. It is not necessary that priming sequences be added at both ends, if only linear amplification is desired. Nor is it

necessary that the priming sequence at the 3' end of a strand be the same as the priming sequence at the 5' end.

The strands are then melted apart 12 and hybridized to a terminal sequence binary sorting array, whose immobilized oligos 14 contain a variable segment 15 and a constant segment 16 which is complementary to the universal priming region 17, including the restored recognition site of the restriction enzyme 16a, 17a. Each strand is at a location dependent upon its variable sequence 100 adjacent to its priming sequence. At this point the array need not be sectioned. The array is then washed to remove unhybridized strands. The entire array is then incubated with DNA polymerase. Consequently, a complementary copy 18 of each hybridized DNA strand is generated by extension of the 3' end of the oligo to which the strand is bound. The array is then vigorously washed to remove the original DNA strands and all other material not covalently bound to the surface (not shown).

The covalently bound copy strands can be amplified. During amplification it is usually desirable that the array be sectioned. The wells are filled with a solution containing universal primers 19, 20, an appropriate DNA polymerase, and the substrates and buffer needed to carry out PCR. The array can, if desired, be sealed with a coversheet, further isolating the wells from each other. PCR is carried out simultaneously in each well of the array. This results in sorting the mixture of strands into groups of strands that share the same terminal oligo sequence, each strand (or each group of strands) being present in a different well of the array and amplified there.

The results of hybridization can be improved by "proofreading", or editing, the hybrids formed, by selectively destroying those hybrids that contain mismatches, without affecting perfect hybrids.

The length of the immobilized oligos in a strand sorting array is chosen to suit the number of strands to be sorted. When sorting strands according to their terminal sequences, the number of different strands obtained in each well equals the number of times that a particular oligo complementary to the variable segment of the immobilized oligo occurs among the termini of

different strands in the mixture. If the number of nucleotides in each variable segment is n, then the total number of such variable sequences is 4^n, and the mean number of different strands in a well is $N/4^n$, where N is the number of different strands in the mixture, provided that nucleotide sequence is random, and that each of the four nucleotides is present in equal proportion. If a random sequence that is the size of an entire diploid human genome (6×10^9 basepairs) is completely digested by a restriction endonuclease that has a hexameric recognition site, then the resulting mixture will contain approximately 3×10^6 strands with an average length of 4,096 nucleotides. If this mixture is then applied to a comprehensive binary array having variable segments eight nucleotides long, then each well will contain, on average, approximately 45 different strands.

Our invention also includes methods for isolating individual strands by sorting them according to the identity of their terminal sequences on sectioned binary arrays. The strands can be from restriction fragments or not, so long as unique priming sequences are added to at least one of the strand's termini, such as by methods described herein. If the number of different strands in a sample is rather small, there is a high probability that after the first stage of sorting, many wells will either not be occupied, or be occupied by only one type of fragment. In the case of a complex mixture of strands (such as from the digestion of an entire human genome), a number of different types of fragments will occupy each well. In that case, the isolation of individual fragments can be achieved by PCR amplifying the strands in each well in the first stage of sorting and then sorting the group of fragments from each well on a fresh sectioned array. After symmetric PCR amplification, each well of the first array will contain copies of the strands that were originally hybridized there, and also their complementary copies.

If the original strands were sorted by their 3' ends, then their copies in a given well will all possess the same 3'-terminal sequence, and their complementary copies will possess the same 5' end. However, the 3'-terminal sequences of the complementary copies of the original strands in each well will be

different (as will be the 5' terminal sequences of the original copies). Therefore, the complementary strands will bind at different locations within the new sectioned array, according to the identity of their own 3'-terminal sequences, and with a high probability, each of them will occupy a separate well, where they can then be amplified.

Alternatively, the second stage of sorting can be carried out according to the identity of the terminal sequences at the other end of each strand. For example, if the strands were sorted in the first stage by their 3' ends (on an array whose immobilized oligos contain upstream constant segments, then the groups of strands from each well in the first array can be sorted in a second stage by their 5' termini (on an array having downstream constant segments). In either procedure, as a result of the second round of sorting, almost all of the different types of fragments are separated from one another (with the exception of virtually identical allelic strands from a diploid genome, which usually have identical termini, and consequently are sorted into the same well). The isolated strands can then be used for any purpose. For example, they can be inserted into vectors and cloned, or they can be amplified and their sequences determined.

Our invention also includes the use of binary arrays for isolating selected strands by sorting according to the identity of terminal sequences. Strands can, for example, be selected that contain particular regions (such as genes) of special interest from a clinical viewpoint. After the relevant portion of a genome has been sequenced, an array can be made using only preselected oligos whose variable segments uniquely match the terminal sequences of the strands of interest, i.e., they would be long enough to uniquely hybridize to the desired strands.

Our invention also encompasses methods that include sorting fragments according to their internal sequences. When so sorting, strands may bind at more than one well. This type of sorting can be useful for a number of applications, such as the isolation of strands that contain particular internal sequence segments (utilizing a sectioned ordinary array), or the sorting of strands according to the identity of variable oligo segments

adjacent to internal restriction sites of a particular type (utilizing a sectioned binary array). The latter approach is useful for ordering sequenced restriction fragments. The sorting of strands by their internal segments on a 3' sectioned ordinary array is useful for the generation of partial strands by virtue of extension of the immobilized oligos.

Our invention includes the sorting, in particular for sequencing, of natural mixtures of RNA molecules, such as cellular RNAs. Establishing messenger RNA sequences is useful, not only for the identification and localization of genes in the genomic DNA, but also for providing information necessary to determine the coding gene sequences (i.e. the exon/intron structure of each gene). Furthermore, the analysis of cellular RNAs in different tissues, at different stages of development, and in the course of a disease, will clarify which genes are active. Usually, RNAs are short enough to be sorted and analyzed without preliminary fragmentation.

III. Preparing partial strands of nucleic acids on sectioned arrays

Our invention includes methods of using sectioned arrays for preparing all possible partial copies of a strand or a group of strands. Preparing complete sets of partials of a strand(s), and sorting the partials by their variable ends is especially useful in a process for determining the sequence of the strand or strands. The preparation of partials is accomplished by either of the following methods: (1) terminally sorting on sectioned binary arrays a mixture of partial strands generated by degradation of a "parental" strand(s) at random; or (2) generating partials on a sectioned ordinary array, through the sorting of a parental strand(s) according to the identity of the strand's internal sequences, followed by the synthesis of (complementary) partial copies of the parental strand(s) by the enzymatic extension of the immobilized oligos, utilizing the hybridized parental strands as templates, and then copying the immobilized partials.

By using comprehensive arrays, it is possible to prepare every possible one-sided partial of a strand.

In the first case (partialing before sorting), a strand, or a double-stranded fragment, or a group of either, carrying terminal priming regions, (these can be a strand or a group of strands sorted on a sectioned binary array as described above), is randomly degraded by a chemical or an enzymatic method, or by a combination of both. Then the mixture of partials is sorted on a sectioned binary array according to the identity of their newly generated termini, essentially as described above for the sorting of full-length strands by their terminal sequences, with new priming sites being introduced at these new termini either before or after sorting. Only those partials that possess both the newly introduced priming site and the already existing priming site (at the opposite end), will be amplified by subsequent PCR. Partials can be sorted according to the identity of a variable sequence at either their 3' termini or their 5' termini. However, as is the case for the sorting of full-length strands, the highest specificity can be achieved by sorting according to the identity of a variable sequence at the 3' termini, and carrying out the sorting on 3' arrays having upstream constant segments, or by sorting according to the identity of a variable sequence at the 5' termini, and carrying out the sorting on 5' arrays having downstream constant segments. In these cases, sorting can be followed by the generation of immobilized (complementary) copies of the sorted partials. The arrays with the immobilized copies can serve as permanent banks of the sorted partials which can subsequently be amplified over and over to generate copies for further use. Following sorting, each well in the array will contain immobilized copies of all of those partials whose variable end is complementary to the variable segment of the immobilized oligo. The other (fixed) end of these partials will be identical to one of the ends of the parental strands. If an oligo segment occurs more than once in a strand, or if it occurs in more than one strand in the group of strands subjected to partialing, then the well will contain a

corresponding number of different partials, all sharing the same sequence at their variable ends.

In the second case (sorting before partialing), partials are prepared directly from the parental strands that are hybridized to a sectioned ordinary array without prior degradation. A strand, or a mixture of strands, is hybridized to a 3' ordinary array. The immobilized oligos are then used as primers for copying the hybridized strands, beginning at the location within each bound strand where hybridization occurred, and ending at the upstream terminus of each bound strand. After extension of the immobilized oligos, the hybridized parental strands are discarded. At this point the wells contain immobilized (complementary) partial strands. The partials in one well all share a 5'-terminal oligo segment that is complementary to a particular internal oligo in the parental strand(s). The partial strands have 3'-terminal sequences that include the complement of the 5'-terminal region of the parental strand(s) (which contains a priming region). Unlike the methods described above for partialing before sorting, the immobilized complementary partials will contain a priming region at only one end and therefore can not be amplified exponentially. However, their linear amplification is possible, with the partials being synthesized as DNAs or RNAs. Where RNA partials are generated, the priming region at the partial copy's 3' terminus contains an RNA polymerase promoter. Synthesis of RNA copies is more efficient than linear synthesis of DNA copies. Alternatively, the synthesized copies can be provided with second priming regions and can then be amplified in an exponential manner by PCR. This approach is illustrated, schematically, in Figure 5.

Figure 5 illustrates the generation of partials for one DNA parental strand 30 on a 3' sectioned ordinary array. First, the strand 30 (many copies, of course) such as obtained from well 13a of sorting array 13, is hybridized to the partialing array 31, a 3' sectioned ordinary array, containing well 31a. The parental strand 30 binds to many different locations within the array, dependent on which oligo segments are present in the strand. A hybrid 32 is formed in each well at the array that contains an

immobilized oligo complementary to a strand's oligo segment. After hybridization, the entire array is washed and incubated with an appropriate DNA polymerase in order to extend the immobilized oligos utilizing the hybridized strand as a template. Each extension product 33 strand is a partial (complementary) copy of the parental strand. Each partial begins at the place 32 in the strand where hybridization occurred and ends at the strand's terminus. The strand preferably terminates at its 5' terminus with a universal priming sequence 17, such as one introduced into all strands when sorting strands on a sectioned binary array as described. This allows for amplification of the partials. That priming sequence can contain a restored restriction site 16a. The parental strand may also contain, if it was previously sorted on a binary sorting array, a priming sequence at its 3' terminus 17, adjacent to the variable sequence 100 that the strand was previously sorted by.

The entire array is then vigorously washed under conditions that remove the parental DNA strands and other material, preferably all, that is not covalently bound to the surface. The areas of the array then contain immobilized strands 33 that are complementary to a portion of the parental strand. The wells can then be filled with a solution containing the universal primer (or promoter complement), an appropriate polymerase, and the substrates and buffer needed to carry out multiple rounds of copying of the immobilized partial strands. The array can then be sealed, isolating the wells from each other, and (linear) copying can be carried out simultaneously in all of the wells in the array.

IV. Surveying oligonucleotides with binary arrays

Our invention includes using binary arrays to survey oligos contained in strands and partials. Binary arrays allow surveying to be improved as compared with ordinary arrays, and they allow new types of selective surveying (such as surveying "signature oligonucleotides").

In surveying, strands first can be randomly degraded into pieces whose average length slightly exceeds the surveyed length. After degradation, each resulting nucleic acid piece is ligated to the same type of oligo (i.e., a constant sequence), that preferably does not occur anywhere in the internal regions of the pieces. For example, the sequence of the added oligo can contain the recognition site of a restriction endonuclease that was used to digest the DNA prior to fragment sorting. The ligation can be carried out in solution prior to hybridization, or after hybridization of the pieces to binary immobilized oligos whose constant segment is complementary to the oligo to be ligated. Preferably, a 3' array is used, having upstream constant segments. The immobilized oligos can then be extended with an appropriate DNA polymerase, using the hybridized nucleic acid pieces as templates. It is preferable that after extension all hybrids have the same length. This can be achieved by employing dideoxynucleotides as substrates for the polymerase, to restrict extension to one nucleotide.

Hybrids can be labeled in both a ligation-dependent and an extension-dependent manner to increase the specificity of hybrid detection. Also, the ligated oligos and the added dideoxynucleotides can be tagged with different labels, for example, fluorescent dyes of different colors. The array is then scanned at two different wavelengths, and only those areas that emit fluorescence of both colors indicate perfect hybrids.

Survey results can be improved further by hybrid proofreading, by destroying hybrids containing mismatches, and by using chemical or enzymatic methods.

V. Use of the oligonucleotide arrays for the sequencing of nucleic acids

The arrays and methods of this invention can be used to determine the nucleotide sequence of nucleic acids, including the sequence of an entire genome, whether it is haploid or diploid. This embodiment requires neither cloning of fragments nor preliminary mapping of chromosomes. It is especially significant that

our method avoids cloning, a labor-intensive and time-consuming approach that is essentially a random search for fragments. In a preferred embodiment a comprehensive collection of whole nucleic acids or fragments is sorted into discrete groups. The sorted nucleic acids are then amplified with a polymerase, preferably by PCR.

Sequencing large diploid genomes, such as a human genome, using the arrays and methods of this invention is shown in Figure 6. We will describe the overall method in general terms. In the embodiment illustrated in Figure 6 an individual's genomic DNA 40 is digested with a restriction endonuclease and sorted by terminal sequences into groups of strands using a 3' sectioned binary sorting array 13, as is described above in Section II and illustrated in Figure 4.

Next, treating each well 13a of the sorting array separately, a complete set of partials is prepared for each group of sorted strands using a sectioned array 31, as is described above in Section III and illustrated in Figure 5. The partials can be generated in any chosen manner to make them detectable.

Then the contents of each well 31a of the partialing array 31 is surveyed using a survey array 42, as is described above in Section IV. Preferably the survey array is a binary array, but an ordinary array may be used. In the embodiment shown in Figure 6, surveying is performed with a sheet 43 containing miniature survey arrays 42 that have been printed in a pattern that coincides with the number and location of the wells 31a. The oligo information obtained can be used, according to our invention, to separately determine the nucleotide sequence of every strand in each group isolated on the sorting array.

To determine the order of the fragments sequenced as illustrated in the embodiment of Figure 6, genomic DNA 40 is digested with at least a second restriction endonuclease and sorted into groups of strands using a 3' sectioned binary sorting array 44, as is described above in Section II and illustrated in Figure 4. The contents of each well 44a of the sorting array 44 is surveyed with special survey arrays 45, 46 that identify "signature oligonucleotides" (described below) in intersite

segments of sorted fragments from different digests. This is done to determine the order of the fragments relative to one another without regard to differences between allelic pairs of fragments. In the embodiment shown in Figure 6 this surveying is performed with printed sheets 47, 48 that have been printed with a pattern of miniature arrays 45, 46.

To allocate the ordered allelic fragments to their respective chromosomes in a diploid organism, fragments are linked according to their allelic differences. In the embodiment illustrated in Figure 6, the strands from selected wells of the sorting array 44 are transferred to a selected well of one of a series of partialing arrays 49, partials are generated, and the partials are surveyed using miniature survey arrays 50 on printed sheets 51. Only the presence of oligos containing allelic differences in the selected partials needs to be determined to link a pair of allelic fragments to their respective neighboring allelic fragments.

When sorting according to the identity of terminal sequences, each strand occupies a particular "address" in the array. It is convenient to think of the address as the oligo sequence within a strand that directs the DNA strand to hybridize to a particular location, i.e., the sequence that is perfectly complementary to the variable sequence of the oligo immobilized at that location. The "address" also identifies the location within the array where the DNA binds.

After sorting, each group of strands is amplified and subjected to partialing. Importantly, the isolation of individual strands is not necessary, because our method allows the nucleotide sequence of each strand in a mixture to be determined. In particular, our method allows the sequences of strands in a well of the sorting array to be determined, separately from mixtures of strands in other wells. In a preferred embodiment, the partialing array is comprehensive in order to obtain all possible one-sided partials (i.e., a comprehensive array). Each group of partials is amplified prior to surveying. Most preferably, the amplification is carried out in such a manner that one

of the two complementary partial strands is produced in great excess over the other.

Each group of partials is surveyed to identify their constituent oligos. Surveying is preferably carried out using binary arrays.

Although not necessary, it is preferable to have the survey arrays be as compact as possible. It is anticipated that surveying will be advantageously accomplished simultaneously for many or all wells of a partialing array by utilizing a sheet on which miniature survey arrays have been "printed" in a pattern that coincides with the arrangement of wells in the partialing array, in a manner similar to that shown in Figures 6 and 7. Referring to Figure 7, partialing array 31, comprising an array of wells 31a, is surveyed using sheet 43, having printed thereon an array of miniaturized survey arrays 42. The pattern of arrays 42 corresponds to the pattern of wells 31a, whereby all wells 31a can be surveyed simultaneously.

Automated photolithography techniques for preparing miniature oligo arrays have been developed [Fodor, S. P., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T. and Solas, D. (1991). Light-Directed, Spatially Addressable Parallel Chemical Synthesis, *Science* 251, 767-773]. The manufacture of miniature arrays on a "chip", for use in surveys also has been reported.

Surveying with comprehensive arrays produces a complete list of oligos contained in the partials in each well of the partialing array. This will reveal all oligos present in all partials in that well. The method of this invention can determine the sequences of the original (parental) fragment strands.

The "partials" referred to in this section are one-sided partial strands that begin at the 5' terminus of a parental nucleic acid strand (the fixed end) and end at different nucleotide positions in the strand (the variable end). Partials are sorted in the partialing array according to the identity of their variable ends, and therefore each partial has a particular "address" within the array. As with sorting arrays, an "address" in a partialing array is the oligo sequence that is present at the variable end of the partial strand and that is complementary

to the variable segment of an immobilized oligo. The "address" also relates to the location within the array where the partial strand is found, since the variable segment of the oligo immobilized in that well is complementary to the oligo at the partial's variable terminus. The "address" also relates to the location within the parental strand of a partial's terminal oligo. The location of this "address oligo" within a parental strand is characterized by an "upstream subset" of oligos that come before it in the parental sequence and by a "downstream subset" of oligos that come after it.

Our method of establishing nucleic acid sequences, for either a single strand or a group of parental strands sorted by their terminal sequences, begins by assembling an "address set" for each address in the partialing array. The "address set" is a comprehensive list of all oligos in all the parental strands which have the address oligo within their nucleotide sequences. The "upstream subset" contains all the oligos that occur upstream (i.e., towards the 5' end) of the address oligo in parental strands that contain the address oligo. The "downstream subset" contains all the oligos that occur downstream (i.e., towards the 3' end) of the address oligo in any parental strands that contain the address oligo. Together the two subsets form the "address set."

The upstream subset of each address can be determined directly from the survey of each well of a partialing array and consists of a list of all the oligos identified as being present in the partial strands in that well. The downstream subset of each address can be inferred by examining the upstream subsets of all the addresses: the downstream subset of a particular address consists of those addresses whose own upstream subset includes that particular address oligo.

The upstream subset and the downstream subset of a particular address, taken together, are an "indexed address set". If an oligo occurs more than once in a strand, it can occur in both the upstream and the downstream subsets of an address. Indexed address sets provide the information required to order the oligos contained in a strand set, as will be described below.

When a mixture of strands is examined, it is also useful to consider an address set without regard to which oligos occur upstream and downstream of an address. This is called an "unindexed address set". Unindexed address sets are decomposable into strand sets by the method of this invention.

We have discovered that when assembling big strand sets whose oligos do not all overlap uniquely, it is advantageous to work with "sequence blocks" rather than with individual oligos. Sequence blocks are composed of oligos that uniquely overlap one another in a given strand set. Two oligos contained in a strand set are said to overlap if they share a terminal (5' or 3') n-1 nucleotide sequence. An overlap is unique if no other oligo than those two in the strand set has this sequence at its termini. Here n is the length (in nucleotides) of each of the two oligos if they are of the same length or, if they are of different length, n is the length of the shorter one. We use unique overlaps to construct sequence blocks from the oligos in a strand set.

The position of each sequence block relative to the others is determined from the distribution of the oligos between the upstream and downstream subsets of every address. This is accomplished by finding, for each of the blocks, which blocks occur upstream, and which blocks occur downstream, of that block by examining the address sets. The address sets are used in order to generate "block sets." The block sets are address sets wherein blocks have been substituted for the oligos that comprise the blocks, including the address oligo. Once the relative position of the sequence blocks has been determined, they can be assembled into the final sequence. The assembly is governed by the following rules: (1) each of the blocks must be used at least once, (2) the blocks must be assembled into a single sequence, (3) the ends of neighboring blocks must match each other (i.e., overlap by an n-1 nucleotide sequence, see above) and (4) the order of the blocks must be consistent with their positions relative to one another, as ascertained from the block sets, as will be clear from the examples.

A sequence block can occur either once in a sequence, or more than once, and this we determine by examining the block sets. If a block occurs more than once in a sequence, it will always be contained in both its own upstream and downstream subsets. On the other hand, if a block occurs only once in a sequence, it may or may not be present in its own upstream or downstream subset. But, if a block is absent from either its upstream subset, or from its downstream set, that block occurs in the strand only once. The relative order of these "unique" blocks can be determined by noting which of them occur in the upstream subset, and which of them occur in the downstream subset, of the others. Once the unique blocks have been ordered relative to each other, the gaps between them are filled with blocks that may be non-unique. However, not every gap can necessarily be filled in with a particular block. There is a range of locations within which each non-unique block (or presumably-non-unique block) can be present. The range for a particular block is determined by noting those blocks that always occur upstream of it, and those blocks that always occur downstream of it. A gap can be filled in if, and only if, there is a block or a combination of blocks, whose outer ends have $n-1$ nucleotide-long perfect sequence overlaps with the ends of the blocks that form the gap. Because at least two overlaps, each of low probability, must occur simultaneously, it is highly unlikely that more than one block, or one combination of blocks, can fill a gap. If a particular block occurs many times in a strand, it will have to be used to fill every gap it matches. This is why, using the method of the invention, it is possible to establish the sequence of a strand without measuring how many times an oligo occurs in the partials. It is only necessary to determine whether an oligo is present or not.

An important aspect of this invention is the ability to sequence a mixture of strands simultaneously. The invention can be used for the determination of fragment sequences from an entire fragmented and sorted genome.

If one strand is being sequenced, all address sets determined from a partialing array will contain the same oligos that

constitute the strand set. The only difference is that some oligos which are downstream in one set may be upstream in another address set. If a mixture of strands have been partialed on a single partialing array, certain addresses will be shared by more than one parental strand. Their address sets will be composite, containing all of the oligos from all of the strands that the address oligo is present in. Addresses that are only found in a particular strand in the mixture, however, will have address sets which only contain oligos from that strand. They are identical to the strand set, and each contain the same oligos. The mixture can contain up to a hundred or so different DNA strands, each of a different length and sequence, as can be obtained with an appropriate sorting array (or set of sorting arrays) and method described above. When a mixture of strands is analyzed on a partialing array, the data obtained by surveying the partials will reflect the diversity of the sequences in the mixture, and will appear to be very complex. However, we have discovered a way to decompose the unindexed address sets obtained by analysis of a strand mixture into their constituent strand sets. Then, as we have described for sequencing a single strand, the oligos in each of the identified strand sets can be grouped into sequence blocks that can be ordered from the information contained in the indexed address sets, as will be clear from the examples.

Unindexed address sets can be either "prime" or "composite." A prime set consists of one strand set; while a composite set consists of more than one. A prime set cannot be decomposed into other address sets, i.e., there is no address set which is a subset of a prime set. Composite sets, however, can usually be decomposed into two or more simpler address sets. Once individual strand sets have been identified, they can each be treated as though they were obtained from an analysis of a homogeneous strand. It is thus possible, in many cases, to sequence all strands in an unknown heterogenous DNA sample without first isolating the strands.

The fragment sequences obtained by the methods outlined above or by any other method can then be put in their correct order using oligo arrays. Assembling restriction fragments into

contiguous sequences can be accomplished by identifying each fragment's immediate neighbors. One method for obtaining this information is to use another restriction enzyme to cleave the same DNA at different positions, thus producing a set of fragments that partially overlap neighboring fragments from the first digest, and then to sequence these fragments. However, it is not necessary to sequence the fragments in the second restriction digest. It is only necessary to uniquely identify overlapping segments in the fragments from alternate restriction digests. This can be done by surveying "signatures".

Signatures can be determined by hybridization of fragment strands to complementary oligo probes. A signature of a fragment may consist of one, two or more oligos, so long as it is unique within the sequence analyzed. Neighboring fragments from one restriction digest can be determined by looking for their signatures in overlapping fragments from an alternate digest.

We have devised a method for identifying neighboring restriction fragments among the list of sequenced fragments that does not require either cloning or sequencing of overlapping fragments. If strands from an alternate digest are sorted, complementary strands of the same fragment will hybridize to different addresses in the sorting array. Whenever intersite segments from two or more fragments of the first digest are present within one fragment of the second digest, then all of these segments will be represented in both complementary strands of that one fragment, and all will be present wherever those strands bind in a sorting array. We identify the segments by obtaining their signatures through hybridization to specialized binary survey arrays. The signatures of intersite segments that occur in one fragment always accompany each other, whereas signatures of distant segments travel independently.

After the fragments from an original (first) restriction digest of a long DNA have been sequenced, the same DNA is digested with a second (different) restriction endonuclease, the termini of the generated fragments are provided with universal priming regions (that also restore the recognition sites at the termini), and the strands are sorted according to particular

internal sequences, namely, a variable sequence adjacent to the recognition site for the first restriction enzyme. The sorting array is a sectioned binary array. It contains immobilized oligos having a variable sequence as well as an adjacent constant sequence that is complementary to the recognition sequence of the first restriction endonuclease. The sorted strands are amplified by "symmetric" PCR, so that in each well where a strand has been bound, copies of the bound strand, as well as complements, are generated. In another embodiment, strands can be sorted according to their terminal sequences on an array whose oligos' constant segments include sequences that are complementary to the recognition site of the second restriction enzyme. This alternative is not detailed, but it corresponds to the embodiment discussed below, but with terminal sorting.

Each strand that hybridizes to the binary sorting array will possess at least two recognition sites for the second restriction enzyme (restored at the strand's termini), and at least one (internal) recognition site for the first restriction enzyme. The segments included between these two types of restriction sites (intersite segments) comprise the overlaps between the two types of restriction fragments, and each intersite segment is thus bounded by any two restriction sites of the two types. It follows, that each of these segments can be characterized by identifying these two restriction sites and variable sequences of preselected length within the segment that are immediately adjacent to each of the restriction sites. The combination of a recognition site (for either the first or the second restriction enzyme) and its adjacent variable oligo we call a "signature oligonucleotide". Every intersite segment can be characterized by two signature oligos (of either type) that bound that segment. The combination of the two signature oligos is defined herein as the intersite segment's "signature".

After strand amplification, the strands in the wells of the sorting array are surveyed to identify the signature oligos of each of the two types. This is carried out by using two types of binary survey arrays. The first has immobilized oligos containing a variable oligo segment and a constant segment that is, or

includes, an adjacent sequence that is complementary to the recognition site for the first restriction endonuclease. The immobilized oligos in the second survey array has a variable oligo segment of preferably the same length as the variable segment of the first specialized survey array, and a constant segment that is, or includes an adjacent sequence that is complementary to the recognition site for the second restriction endonuclease. The constant oligo segments in these arrays can be located either upstream or downstream of the variable oligo segments, resulting in the surveying of either the downstream or the upstream signature oligos in each strand of the intersite segments being surveyed. In a preferred embodiment the constant oligo segments are upstream, and the immobilized oligos have free 3' ends, so that they can be extended by incubation with a DNA polymerase. From the oligo information that is obtained, the sequenced fragments can be ordered relative to one another.

In our method, the uniqueness of a signature is achieved by surveying "half signatures" (signature oligonucleotides) on two relatively small survey arrays. If the variable segments in the arrays are 8-nucleotide-long, the number of areas in the two arrays is approximately 130,000, or approximately 100,000,000 times smaller than the single array that would be needed for detecting the same size signature (28 nucleotides).

If a diploid genome (such as a human genome) is sequenced, the ordered fragments will appear as a string of unlinked pairs of allelic fragments. What remains unknown is how the allelic fragments in each pair are distributed between the homologous (sister) chromosomes that came from each parent. Allocation of the allelic fragments to these "chromosomal linkage groups" requires knowledge of which fragment in each pair is linked to which fragment in a neighboring pair.

We have developed a method that uses arrays for allocating allelic fragments to chromosomes, irrespective of what method was used for sequencing and ordering the fragments. The linkage of fragments in neighboring pairs can be achieved by sequencing a restriction fragment ("spanning fragment") from an alternate digest that spans at least one allelic difference in each pair.

Since the sequences of the allelic fragments are known, there is no need to sequence the spanning fragment. Instead, one can simply determine which oligos that harbor allelic differences accompany one another in the spanning fragment, i.e., which oligos occur in the same chromosome. This can be accomplished by surveying, at a selected address in a partialing array, partials generated from a selected group of restriction fragments from an alternate digest. A group of restriction fragments is selected that contains a spanning fragment, and an address in a partialing array is selected that encompasses a difference in one of the neighboring allelic pairs.

Since the sequence of every fragment is known, it is possible to choose an alternate restriction fragment that spans the allelic differences in the neighboring pairs. A spanning restriction fragment, in fact, may already be present at a particular address in one of the sorting arrays used to sort alternate digests during the ordering procedure.

In this method, sorted strands are melted apart, and the mixture is hybridized to a particular well in the partialing array, whose address corresponds to one of the allelic oligos. Two different wells are selected, each with an address that corresponds to an oligo that harbors a differenct allelic oligonucleotide After amplification of the partial strands, the oligos in the two wells are identified with a survey array. Examination tells which fragments are on the same chromosome.

Since allelic differences occur roughly once every 1,000 basepairs in the human genome, most allelic fragments resulting from digestion with a restriction enzyme recognizing a hexameric sequence (resulting in about 4,096 average length) will differ from each other. If the variable oligo segments in the survey arrays are made of octanucleotides, then each allelic nucleotide substitution will give rise to eight different oligos in each of the allelic fragments. However, using our method, inspection of only one address in the partialing array is sufficient to reveal the linkage of the corresponding reference oligo to any one of the eight oligos that encompass the nucleotide substitution that occurs in the neighboring fragment on the same chromosome.

Therefore, only one address in the partialing array is needed to reveal the linkages between two neighboring allelic pairs. Thus, 65,536 linkages can be determined on a single comprehensive partialing array made of variable octanucleotides. With this method, only 10 to 20 of these arrays would be needed to complete the assembly of an entire diploid human genome that has been fragmented by a restriction endonuclease with a hexameric recognition site.

Computational methods can be developed to minimize or eliminate errors that occur during partialing and surveying, by taking advantage of the high redundancy in the data. Such methods should take into account the following aspects of a preferred sequencing procedure: the sequence of every fragment is independently determined four times (by virtue of each strand and its complement being present at two different addresses in the sorting array); each strand set is determined in as many trials as the number of different oligos in that strand; every nucleotide in a strand is represented by as many different oligos as the length (of the variable segment) of the immobilized oligos in the survey array; the locations where a particular block can occur in a sequence are limited by the distribution of the blocks among the upstream and downstream subsets of each pertinent address; and the edges of a block must be compatible with the edges of each gap where that block is inserted.

Using our genome sequencing method, one can use throughout essentially the same technology, i.e., hybridization of oligo probes and the amplification of nucleic acids by the polymerase chain reaction, both of which are well-studied, common laboratory techniques. The entire procedure can be performed by a specially designed machine, resulting in huge reductions in time and cost, and a marked improvement in the reliability of the data. Many arrays could be processed simultaneously on such a machine. The machine most preferably should be entirely computer-controlled, and the computer should constantly analyze intermediate results. As stated above, used arrays can be stored, both to serve as a permanent record of the results, and to provide additional

material for subsequent analysis or for manipulating the sequenced strands and partials.

Analysis of an individual's genomic DNA provides the complete nucleotide sequence of that individual's diploid genome. The genes and their control elements are allocated into chromosomal linkage groups as they appear in a single living organism. The sequence will describe an intact, functioning ensemble of genetic elements. This complete sequencing provides the ability to compare genomes of individuals, thereby enabling biologists to understand how genes function together and to determine the basis of health and disease. The genomes of any species, whether haploid or diploid, can be sequenced.

The invention can be used not only for DNA's but as well for sequencing mixtures of cellular RNAs.

The invention is also useful to determine sequences in a clinical setting, such as for diagnosis of genetic conditions.

VI. Manipulating Nucleic Acids on Sectioned Arrays

Our invention also includes using sectioned arrays for introducing site-directed mutations into sequenced nucleic acids, including the introduction of nucleotide substitutions, deletions and insertions. This can be carried out in a massively parallel fashion. In one embodiment, a partial whose variable end has been deprived of a priming region, is ligated to the free terminus of an immobilized oligo that contains the mutation to be introduced. In another procedure, where the purpose of mutagenesis is to introduce a single-nucleotide substitution, then the substituting nucleotide can be added directly to the variable end of the partial. In both cases, the modified partials or their complementary copies are used to synthesize a mutant strand utilizing as a template either the complementary parental strand (i.e., from which the partials were generated) or a longer complementary partial, or any other strand or partial that encodes the missing region. The fixed end of the mutant partial is provided with a priming region that is different from the corresponding priming region of the template strand. Therefore, only mutant strands are capable of subsequent amplification by

PCR. A single array can be used either to mutate many single positions in a gene, or to introduce mutations in many genes in one procedure.

Sectioned arrays can also be used for the massively parallel testing of the biological effects of the introduced mutations. For example, parallel coupled transcription-translation reactions can be carried out in the wells of a sectioned array following amplification of the mutant strands. It is thus possible to determine simultaneously, on the same sectioned array, the effects of many different amino acid substitutions on the structure and function of a protein.

VII. Examples

1. Sorting nucleic acids or their fragments on a binary oligonucleotide array whose immobilized oligos have free 3' termini, with constant upstream segments --

This method allows the immobilized oligos to serve as primers for copying bound strands, resulting in the formation of complementary copies covalently linked to the array.

1.1. Sorting restriction fragments according to their terminal sequences, following the introduction of terminal priming regions --

DNA is digested using a restriction endonuclease. Recognition sites for the restriction endonuclease are restored in solution by introducing terminal extensions (adaptors) that contain a sequence which, together with the restored restriction site, form a universal priming region at the 3' terminus of every strand in the digest. This priming region is later used for amplification by PCR. After melting fragments, the strands are sorted on a sectioned binary array. A sequence complementary to the generated priming region serves as both the constant segment of the immobilized oligos and as the primer for PCR amplification of the bound strands.

DNA to be analyzed is first digested substantially completely with a chosen restriction endonuclease, and the fragments

obtained are then ligated to synthetic double-stranded oligo adaptors. The adaptors have one end that is compatible with the fragment termini. The other end is not compatible with the fragments' termini. The adaptors can therefore be ligated to the fragments in only one orientation. The adaptors' strands are non-phosphorylated, which prevents their self-ligation. The strands in the restriction fragments have their 5' termini phosphorylated which results from their cleavage by a restriction endonuclease. This favors the ligation of the adaptors by a DNA ligase (such as the DNA ligase of T4 bacteriophage) to the restriction fragments, rather then to each other. Since DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 3' hydroxyl and phosphorylated 5' termini in a double-stranded DNA, the phosphorylated 5' termini of the fragments are ligated to the adaptor strand whose 3' end is at the compatible side of the adaptor. The 3' termini of the fragments remain unligated. A DNA polymerase possessing a 5'-3' exonuclease activity (such as DNA polymerase I from *Escherichia coli* or Taq DNA polymerase from *Thermus aquaticus*) is then used to extend the 3' ends of the fragments, utilizing the ligated oligo as a template, concomitant with displacement of the unligated oligo. To make the ligated oligo resistant to the 5'-3' exonuclease, the ligated oligo can be synthesized from α-phosphorothioate precursors.

Although the oligo adaptors are provided in great excess during the ligation step, there is still a low probability that two restriction fragments will ligate to one another, rather then to the adaptor. To prevent this, the ligation products can again be treated with the restriction endonuclease used to generate the fragments, in order to cleave the formed interfragment dimers. The endonuclease will not cleave the ligated adaptors if they are synthesized from modified precursors (such as nucleotides containing N^6-methyl-deoxyadenosine), which are known and currently commercially available [e.g., from Pharmacia LKB]. Resistance of the ligated adaptors to digestion by the restriction endonuclease can be increased further if the ligated oligo is synthesized from phosphorothioates, and if phosphorothioate analogs of the nucleo-

side triphosphates are used as substrates for extension of the 3' termini.

After the priming regions have been added, the complementary strands are melted apart, such as by increasing temperature and/or by introducing denaturing agents such as guanidine isothiocyanate, urea, or formamide. The resulting strands are hybridized to a binary sorting array, such as by following a standard protocol for the hybridization of DNA to immobilized oligos. Hybridization is performed so that formation of only perfectly matched hybrids is promoted. The hybrids have a length which is equal to that of the immobilized oligos. The immobilized oligos are attached to the array at their 5' termini and contain constant restriction site segments adjacent to a variable segment of predetermined length. Each strand will be bound to the array at its 3' terminus. Its location within the array will be determined by the identity of the oligo segment that is located in the strand immediately upstream from the restored restriction site at its 3' end, and that is complementary to the variable segment of the immobilized oligo to which it is bound. After hybridization and washing away all unbound material, the entire array is incubated with a DNA polymerase, such as Taq DNA polymerase deoxyribonucleotide 5' triphosphates or the DNA polymerase of bacteriophage T7, and substrates. As a result, the 3' end of each immobilized oligo to which a strand is bound will be extended to produce a complementary copy of the bound strand. The array is vigorously washed. The wells are then filled with a solution containing universal primer, an appropriate DNA polymerase, and the substrates and buffer needed to carry out PCR. The array is then sealed, isolating the wells from each other, and exponential amplification is carried out, preferably simultaneously, in each well.

1.2. Sorting restriction fragments according to their terminal sequences, with 3' and 5' terminal priming regions being introduced, one before and one after strand sorting --

This procedure consumes larger amounts of enzymes and substrates than the procedure described in Example 1.1, however,

only those strands that are correctly bound to the immobilized oligos acquire both priming regions necessary for PCR. The possibility that non-specifically bound strands will be amplified is minimized. Furthermore, different priming regions can be introduced at different termini of a strand. It then becomes possible to: (1) perform "asymmetric" PCR, where only one of the complementary strands is accumulated in significant amounts, and remains single-stranded: (2) introduce a transcriptional promoter into only one of the priming regions, in order to be able to obtain RNA transcripts of only one strand (without also producing its complement; (3) differentially label complementary strands; and (4) avoid self-annealing of the strand's terminal segments that can interfere with primer hybridization and lower PCR efficiency.

In this example, digestion of DNA, adaptor ligation and re-digestion of fragments are carried out as described in Example 1.1, above. The 3' ends of the restriction fragments, however, are not extended by incubation with DNA polymerase. Instead, the strands ligated at their 5' ends to adaptors are melted apart from their unextended complements and hybridized to a binary array. The array contains immobilized oligos that are pre-hybridized with shorter complementary 5'-phosphorylated oligos that cover (mask) the immobilized oligos except for a segment which includes a variable region and a region complementary to the portion of the restriction site remaining at the fragments' (unrestored) 3' end. The masked region includes the rest of the restriction site and any other constant sequence, such as may be included in a priming region. Hybridization is carried out under conditions that promote the formation of only perfectly matched hybrids which are the length of the unmasked segment of the immobilized oligo. After washing away the unbound strands, the strands that remain bound are ligated to the masking oligos by incubation with DNA ligase. The correctly bound strands thus acquire a priming region at their 3' end, in addition to the priming region they already have at their 5' end. The two priming regions preferably correspond to different primers. The array is then washed under appropriately stringent conditions to

remove all nucleic acids except the immobilized oligos and the ligated strands hybridized to them.

1.3. Sorting RNAs according to their terminal sequences -- Mature eukaryotic mRNAs share structural features that can help in their manipulation using arrays. All have a "cap" structure on their 5' end, and most also possess a 3'-terminal poly(A) tail, which is attached posttranscriptionally by a poly(A) polymerase. Because there are usually no long oligo(A) tracts in the internal regions of cellular RNAs, the poly(A) tail can serve as a naturally occurring terminal priming sequence in sorting. The size of mRNAs (several thousand nucleotides in length) allows them to be amplified and analyzed directly, without prior cleavage into fragments.

There are known methods for preparing essentially undegraded total cellular RNA. Total cellular RNA is converted into complementary DNA (cDNA) using an oligo(dT) primer and a reverse transcriptase or *Thermus thermophilus* DNA polymerase. Then, omitting second strand synthesis, single-stranded cDNAs (which possess oligo(dT) extensions at their 5' end and variable 3' termini) are sorted according to their 3'-termini on a sectioned binary array and are ligated there to pre-hybridized adaptors of a predetermined sequence that are complementary to the immobilized oligos' constant sequence, and that introduce into a cDNA molecule the 3'-terminal priming site. The cDNA is amplified, using two primers for PCR: oligo(dT) and an oligo complementary to the adaptor.

2. Preparing partial strands of nucleic acids on oligonucleotide arrays --

There are two aspects to this procedure: first, the generation of partial strands (partials), and second, the sorting of partials according to their terminal oligo segments. All of the embodiments described below are based on the following principle: in generating partials from a strand, one of the original strand ends is preserved (it will be referred to as the "fixed" end), whereas the other end is truncated to a different extent in the

various partials (it will be referred to as the "variable" end). Although either the 5' or the 3' end of the original strand can serve as the fixed end, it is preferable that the 5' end be fixed. If amplification of sorted partials is desirable, it is preferable that the 5' end of the original strand, i.e., the fixed end, be provided with a priming region prior to partialing by any of the methods described above, and that partialing be carried out on a sectioned array. Either an individual strand or a mixture of strands can be subjected to a partialing; however, if the mixture is very complex (such as a restriction digest of a large genome), it is desirable that the mixture first be sorted into less complex groups of strands, as described above. The groups of strands used for preparing partials should essentially be devoid of contaminating strands; therefore, sorting by terminal sequences is preferable for the preliminary sorting. If preliminary sorting is performed, the strands will already contain terminal priming regions necessary for amplification of the partials. Partialing can be performed on either DNA or RNA, the final product being either DNA or RNA, in either a double-stranded or a single-stranded state.

2.1. Methods employing enzymatic cleavage of DNA fragments --

The purpose of the cleavage is to produce a set of partials of every possible length; therefore, DNA should be cleaved as randomly as possible, and to the extent that there is approximately one cut per strand. Deoxyribonuclease I (DNase I) cleaves both double-stranded and single-stranded DNA; however, double-stranded DNA is preferable as the starting material for preparing partials because of its essentially homogeneous secondary structure, so that every segment of a DNA molecule is equally accessible to cleavage. Double-stranded DNA fragments are produced as a result of "symmetric" PCR that can be carried out when sorting strands. An advantage of using DNase I is that it produces fragments with 5'-phosphoryl and 3'-hydroxyl termini, that are suitable for enzymatic ligation.

After cleavage of the double-stranded DNA fragments, DNase is removed, e.g., by phenol extraction. The (partial) strands are then melted apart and are hybridized to a sectioned binary array, wherein the immobilized oligos are pre-hybridized with shorter complementary 5'-phosphorylated oligos of a constant sequence that cover (mask) the immobilized oligos except for a segment that consists of a variable sequence. Hybridization is carried out under conditions that favor the formation of perfectly matched hybrids of a length that is equal to the length of the unmasked (variable) segment of the immobilized oligo, and that minimize the formation of imperfectly matched hybrids. After washing away unbound strands, the bound strands are ligated to the masking oligos by incubation with a DNA ligase. The ligated masking oligos will themselves serve as the second (3'-terminal) priming region of a partial strand. (All the partials of a strand will share the same 5' priming sequence that had been introduced into the strand before generation of the partials). If restriction fragments are to be partialed that possess some restriction site at their termini and do not possess this site internally, it is preferable that the 3' terminal priming region added to the partials include that site. This increases the specificity of terminal priming during subsequent amplification of the partials by PCR. Subsequent extension, washing, and amplification steps are as described in Example 1.1. If the partials are prepared for the purpose of sequence determination, asymmetric PCR can be performed. Alternatively, an RNA polymerase promoter sequence can be included in one of the two primers, and amplified DNA is then transcribed to produce multiple single-stranded RNA copies of one of the two complementary partial strands.

2.2. Methods employing chemical degradation of DNA --

These methods are applicable to both double-stranded and single-stranded nucleic acids. Chemical degradation is, in most cases, essentially random. It can be performed under conditions that destroy secondary structure, and the small size of the

modifying chemicals makes the chemicals readily accessible to nucleotides in secondary structures.

Both base-nonspecific reagents and base-specific reagents can be used. In the latter case, after base-specific cleavage is performed separately with several portions of the sample, the portions are mixed together to form a set of all possible partial DNA lengths. The main drawback to chemical cleavage is that the location of the terminal phosphate groups on the fragments is opposite to what is required for enzymatic ligation: 5'-hydroxyl and 3'-phosphoryl groups are produced in most cases. To overcome this problem, enzymatic dephosphorylation of 3' ends can be carried out.

2.3. Method of preparing partials directly on a sectioned array, without prior degradation of nucleic acids --

In this embodiment, the generation of partials and their sorting according to the identity of the sequences at their variable ends occur essentially in one step. First, a strand or a group of strands (if double-stranded nucleic acid is used as a starting material, the complementary strands are first melted apart), is directly hybridized to a sectioned ordinary array, whose oligos only comprise variable sequences of a pre-selected length, and that are immobilized by their 5' termini. Optimally, hybridization is carried out under conditions in which hybrids can only form whose length is equal to the length of the immobilized oligo. If the array is comprehensive, then a hybrid is formed somewhere within the array for every oligo that occurs in a DNA's sequence. After hybridization, the entire array is washed and incubated with an appropriate DNA polymerase in order to extend the immobilized oligo, using the hybridized strand as a template. Each product strand is a partial (complementary) copy of the hybridized strand. Each partial begins at the place in the strand's sequence where it has been bound to the immobilized oligo and ends at the priming region at the 5' terminus of the strand. If a priming region has not been introduced at the strand's 5' end before partialing, it can be generated at this step, after the hybrids that have not been extended, are elimi-

nated by washing. This can be done either by ligating the 5' end of the bound strand to a single-stranded oligoribonucleotide adaptor, or by tailing the immobilized partial copy with a homopolynucleotide. The entire array is vigorously washed under conditions that remove the original full-length strands and essentially all other material not covalently bound. Subsequent amplification of the immobilized partials can be carried out in different ways, dependent on whether it is desired to use linear or exponential amplification.

Exponential copying results in the generation of partials and their complements. For a strand to be exponentially amplified by PCR, both of its termini should be provided with a priming region, preferably different priming regions. The immobilized (complementary) partial contains only one (3'-terminal) priming region, and a complementary copy produced by linear copying would also have only one priming region (on its 5' end). For RNA copies to have a priming region at their 5' ends, the immobilized partial should have been provided with an RNA polymerase promoter downstream of its 3' terminal priming region using the methods described herein. The second priming region that is needed for exponential amplification can be introduced at the 3' ends of the complementary copies as follows.

(a) The 3' termini of RNA copies can then be ligated to oligoribonucleotide or oligodeoxyribonucleotide adaptors which are phosphorylated at their 5' end and whose 3' end is blocked. Exponential PCR can be performed by utilizing the two primers that correspond to the two priming regions, and then incubating with Tth DNA polymerase.

(b) If the amplified copies are DNA, they can be transferred, such as by blotting, (after melting them free of the immobilized partial) onto a binary array that is a mirror copy of the first array in the arrangement of the variable segments of its immobilized oligos. The constant segments of this binary array are pre-hybridized to masking oligos whose ligation to the 3' termini of the transferred DNAs (by DNA ligase) results in generation of the second priming region to permit exponential PCR.

In methods (a) and (b), both priming regions preferably contain, when applicable, the recognition sequence of the restriction endonuclease that was used to digest the genomic DNA before full-length strand sorting, and which had thus been substantially eliminated from the strands' internal regions.

(c) If partials are surveyed only for oligos that occur in one complementary strand (such as detecting only parental oligos), either only one of the two different primers should be labeled, or the primers should be labeled differently. It is also possible to use labeled substrates during asymmetric PCR.

3. Surveying oligonucleotides with binary arrays --

Surveying oligo content can be carried out in the different embodiments of the invention by hybridization of strands (or partials) to an ordinary array, followed by detection of those hybridized. However, the signal-to-noise ratio is not high enough to always avoid ambiguous results. The most significant problem is inability to sufficiently discriminate against mismatched basepairs that occur at the ends of hybrids. That hampers analysis of complex sequences. The use of binary arrays helps to overcome this problem.

Binary arrays are also useful for surveying longer oligos than are easily surveyed on an ordinary array (e.g., signature oligos) without increasing the size over that of an ordinary array.

Immobilized oligos in a binary survey array can have either free 5' or 3' ends, and the constant segment can be either upstream or downstream. In most cases, it is preferable that the 3' ends of immobilized oligos be free, and that their constant segments be upstream.

Surveying can utilize sectioned arrays. However, the use of plain arrays is preferable because they are less expensive and more amenable to miniaturization. The following methods are based on the use of plain binary arrays and involve fragmentation of the strands or partials prior to surveying.

3.1. Comprehensive surveys of DNA strands --

Every oligo present in a strand or in a partial, or in a group of strands or partials, is surveyed. If a survey of partials is performed in order to establish nucleotide sequences, it is preferable that each partial be represented by the same sense copies. Thus, there should be only one of the complementary strands in a sample or the complementary strands should be differentiable, e.g., one strand should produce either no detectable signal or a weaker signal. This can be accomplished by amplifying the partials linearly or by the use of asymmetric PCR.

DNA strands (or partials) to be surveyed are preferably digested with nuclease S1 under conditions that destabilize DNA secondary structure. The digestion conditions are chosen so that the DNA pieces produced are as short as possible, but at the same time, most are at least one nucleotide longer than the variable segment of the oligos immobilized on the binary array. If the surveyed strands or partials have been previously sorted and amplified on a sectioned array, this degradation procedure can be performed simultaneously in each well of that array. Alternatively, if it is desired to store that array as a master for later use, the array can be replicated by blotting onto another sectioned array. The DNA is then amplified within the replica array by (asymmetric) PCR prior to digestion with nuclease S1.

After digestion, the nuclease is inactivated by, for example, heating to 100°C, and the DNA pieces are hybridized to an array whose immobilized oligos' constant segments are prehybridized to 5'-phosphorylated complementary masking oligos. Preferably, the constant segment contains a restriction site that has been eliminated from the internal regions of the strands prior to sorting and is long enough so that its hybrid with the masking oligo is preserved during subsequent procedures.

The array is incubated with DNA ligase to ligate the masking oligos to only those hybridized DNA strands (or partials) whose 3' terminal nucleotide is immediately adjacent to the 5' end of the masking oligo, and matches its counterpart in the immobilized oligo. DNA ligase is especially sensitive to mismatches at the junction site.

After all non-ligated DNA pieces have been washed away under much more stringent conditions that were used during hybridization, the immobilized oligos are extended by incubation with a DNA polymerase, preferably by only one nucleotide, using the protruding part of the ligated DNA piece as a template, and preferably using the chain-terminating 2',3'-dideoxynucleotides as substrates. Extension is only possible, if the 3'-terminal base of the immobilized oligo forms a perfect basepair with its counterpart in the hybridized DNA piece. The use of the dideoxynucleotides ensures that all hybrids are extended by exactly one nucleotide and that all are of the same length. The array is then washed under conditions sufficiently stringent to remove unextended hybrids.

3.2. Detection of hybrids --

Hybrids can be detected by a number of different means. Unlabeled hybrids can be detected by using surface plasmon resonance techniques, which currently can detect 10^8 to 10^9 hybrid molecules per square millimeter. Alternatively, hybrids can be conventionally labeled, such as with radioactive or fluorescent groups. Fluorescent labels are convenient.

To ensure the lowest level of background labeling, it is preferable to label hybrids in a manner such that its detection is dependent on the success of both a ligation and an extension step. This can be accomplished within the scheme of oligo surveying by labeling the masking oligos, and the 2',3'-dideoxynucleotides used for the extension with fluorescent dyes possessing different emission spectra. The array can then be scanned at different wavelengths, corresponding to the emission maxima of the two dyes, and only signals from those areas that emit fluorescence of both colors are taken as a positive result.

After hybrids are extended (concomitant with labeling) and edited, the array is thoroughly washed to remove unincorporated label, destroy unextended hybrids, and discriminate one more time against mismatched hybrids that might have remained. A preferred method is to wash the array at steadily increasing temperature, with the signal from each area being read at a pre-determined

time, when the conditions ensure the highest selectivity for the particular hybrid that forms in that area. Other conditions (such as denaturant and/or salt concentration) can also be controlled over time. The fluorescence pattern can be recorded at predetermined time intervals with a scanning microfluorometer, such as an epifluorescence microscope.

4. Determination of the nucleotide sequences of strands in a mixture when each strand possesses at least one oligo that does not occur in any other strand in the mixture --

Figures 8 to 11 depict the determination of the sequences of two mixed strands using the methods of the invention. The example demonstrates the power of the invention to identify all the oligos present in a strand (i.e., its strand set) when it possesses at least one oligo that does not occur in any other strand in the mixture. In particular, the example demonstrates: (a) how the data obtained by surveying the partial strands generated from a mixture of strands and sorted by their variable termini (i.e., the upstream subset of each address) and the inferred downstream subset of each address (which together form the indexed address sets) are used to construct the unindexed address sets; and (b) how the unindexed address sets are compared to each other to identify prime sets. The example also demonstrates how the oligos contained in a strand set are assembled into the sequence of the strand, even though the primary data is obtained from a mixture. In particular, the example demonstrates: (a) how oligos in a strand set are assembled into sequence blocks; (b) how the contents of the indexed address sets are filtered so that only information pertaining to the oligos in a particular strand set remains; (c) how this filtered data is re-expressed in terms of the sequence blocks that are contained in that particular strand; (d) how information in the resulting "block sets" is used to identify those blocks that definitely occur only once in the strand ("unique blocks") and to identify those that can potentially occur more than once; (e) how information in block sets of unique blocks is used to determine the relative order of the blocks that occur only once in the strand;

(f) how the information in the block sets limits the positions at which the other blocks can occur (relative to other blocks); and (g) how a consideration of the sequences at the ends of blocks, in combination with a consideration of the relative positions of the blocks, leads to the unambiguous determination of the complete sequence of the strand. This example also illustrates: (a) how oligos that occur more than once in a strand are identified and located within the sequence, even though the survey data contain no information as to the number of times a particular oligo occurs in a partial or a mixture of partials having the same terminal oligo; and (b) how the sequences of different strands in a mixture can be determined separately, despite the fact that many of the oligos occur in more than one strand.

Figure 8a shows the sequences of two short strands (parental strands) that are assumed to be present in a mixture (with no other strands). It is assumed that complete sets of partials have been generated from this mixture, and that each set of partials has been separately surveyed, with the partials sharing the same address oligo being surveyed together. For the purpose of illustrating the method of analyzing the data, it is assumed that the address oligos and the surveyed oligos are three nucleotides in length. In practice, longer oligos should be used. However, for illustration it is easier to comprehend an example based on trinucleotides. The same methods of analyzing the data apply when longer oligos are surveyed, when much longer strands are in the mixture, and when the mixture contains many more strands.

Figure 8b shows the upstream subsets determined by surveying and the downstream subsets inferred (i.e., Figure 8b shows indexed address sets). The address oligos (bold letters) are listed vertically in the center of the diagram. The oligos listed horizontally to the left of each address oligo are those oligos that were detected in a survey of the partials at that address (the upstream subset). The oligos listed horizontally to the right of each address oligo are those inferred from the upstream subsets to occur downstream of that address oligo (the downstream subset). For example, oligo "ACC" is contained in the

upstream subset of the address oligo "CCT". This means that oligo "CCT" occurs downstream of oligo "ACC" in at least one strand in the mixture. Therefore "CCT" is inferred to be in the downstream subset of address set "ACC". The remaining downstream oligos in all of the address sets are similarly inferred. Note that an address oligo is a member of its own upstream and downstream subsets.

After the indexed address sets of all addresses in the parental strands have been determined (as shown in Figure 8b), the information is organized into unindexed address sets (Figure 8c), having no division into downstream and upstream subsets, but merely listing, for each address oligo, those oligos that occur in either the upstream or downstream subset (or in both). In Figure 8c, the address oligos (bold letters) are listed vertically on the left side of the diagram. Note that the address oligo is a member of its own unindexed address set.

Unindexed address sets are grouped together according to the identity of the oligos they contain (Figure 8d). Unindexed address sets that contain an identical set of oligos are grouped together. It can be seen that three groups of address sets are formed in this example. The groups are identified by the Roman numerals (I, II, and III). The address oligos of each group (for example, CTA, GTC, and TCC in group II) always occur together in a strand and can occur together in more than one strand.

Each group of identical address sets is then compared to all other groups of identical address sets to see if its common address set appears to be a prime by seeing whether any other address set is a subset of it. For example, in Figure 8d, the address set common to group III is not a prime address set, because the address set common to group I is a subset of the address set common to group III. However, the address set common to group I and the address set common to group II appear to be prime address sets.

Each putative prime address set is then tested to see if it is a strand set by examining all the address sets that contain all of the oligos that are present in it. For example, in Figure 9a, all the address sets that contain all the oligos present in

the putative prime address set common to group I are listed together (namely the address sets contained in groups I and III). The address oligos are shown in bold letters on the left side of the diagram, and the groups are identified by Roman numerals. The address set common to group I is indeed a prime address set (and therefore it contains a single strand set) because a list of the eleven oligos that are found in every address set in the diagram (they are seen as full columns) is identical to the list of eleven addresses on the left side of the diagram. Similarly, Figure 8b shows why the address set common to group II is also a prime set. The twelve oligos common to every address set in the diagram are all found in the list of twelve addresses on the left side of the diagram. Had either of these putative prime address sets not turned out to be a prime set (by the criterion described above), then it would have been identified as a pseudo-prime address set, and further analysis would have been required to decompose it into its constituent strand sets.

Once the strand sets in a mixture have been identified, the oligos in each strand set can be assembled into the strand sequence in a series of steps, as illustrated in Figure 10 (which utilizes the strand set determined in Figure 9a).

First the oligos in the strand set are assembled into sequence blocks. A sequence block contains one or more uniquely overlapping oligos. Two oligos of length *n*, uniquely overlap each other if they share an identical sub-sequence that is *n*-1 nucleotides long and no other oligos in the same strand set share that sub-sequence. For example, for the strand set shown in Figure 10a, the oligos "CAT" and "ATG" share the sub-sequence "AT" which does not occur in other oligos. These two oligos therefore uniquely overlap to form the sequence block "CATG", as shown in Figure 10b. Similarly, oligo "TGG" uniquely overlaps oligo "GGT" by the common sub-sequence "GG", and oligo "GGT" also uniquely overlaps (on its other end) oligo "GTA" by the common sub-sequence "GT". Thus, the three oligos ("TGG", "GGT", and "GTA") can be maximally overlapped to form sequence block "TGGTA". In forming sequence blocks, the following rule is adhered to: two oligos can be included in the same block if they

are the only oligos in the strand set to possess their common sub-sequence. Thus, "ATG" does not uniquely overlap "TGG", because the strand set contains a third oligo, "TTG", that shares the common sub-sequence "TG". If, following these rules, an oligo does not uniquely overlap any other oligo, then a sequence block consists of only that oligo. For example, "TAA" forms its own block. Following the above rules, the eleven oligos that occur in strand set A can be assembled into four sequence blocks.

Second, the data contained in the indexed address sets shown in Figure 8b are filtered to remove extraneous information that does not pertain to strand set A. Figure 10c shows the resulting filtered address sets. All address sets whose address oligo is not one of the oligos in strand set A are eliminated. In addition, all oligos that are not members of strand set A are removed from the upstream and downstream subsets of the remaining address sets. The resulting filtered address sets are then grouped together according to the oligos that are contained in each block. For example, the filtered address sets for address oligos "CAT" and "ATG" have been grouped together in Figure 10c because these two oligos are contained in sequence block "CATG". In Figure 10c, the address oligos found in the same block are identified by rectangular boxes. In addition, oligos that occur in the same block are grouped together within each upstream and downstream subset.

Third, the filtered address sets are converted into block sets, as shown in Figure 10d. In a block set, the information from different address sets is combined. Instead of a different horizontal line for each filtered address set that pertains to a particular block, the information in all of the address sets that pertain to that particular block is combined into a single horizontal line. For example, in Figure 9c, five different filtered address sets pertain to sequence block "TACCTTG". In Figure 10d, these five lines are combined into a single line in which the address oligos are replaced by an "address block", shown as "TACCTTG" surrounded by a bold box. Similarly, the upstream oligos are replaced by upstream blocks, and the downstream oligos are replaced by downstream blocks. In substituting

sequence blocks for the upstream (or downstream) oligos that are contained in the filtered address sets for a given address block, the following rule is adhered to: a sequence block only occurs in the upstream subset (or in the downstream subset) of an address block, if every oligo that is contained in that address block occurs in the upstream (or in the downstream) subset of every filtered address set that pertains to that address block. For example, sequence block "CATG" occurs in the upstream subset of address block "TACCTTG" because oligos "CAT" and "ATG" occur in the upstream subset of address oligos "TAC", "ACC", "CCT", "CTT", and "TTG".

Often, a sequence block does not occur in its own upstream or downstream subset. For example, sequence block "CATG" does not occur in the upstream or downstream subset of its own block set (i.e., in block set "CATG"), because oligo "ATG" is not present in the upstream subset of address set "CAT" and oligo "CAT" is not present in the downstream subset of address set "ATG". When a sequence block does not occur in its own upstream or downstream subset, this indicates that that sequence block occurs only once in the nucleotide sequence of that strand. However, a sequence block may occur in both the upstream subset and in the downstream subset of its own block set. For example, sequence block "TGGTA" occurs in both the upstream subset and in the downstream subset of block set "TGGTA". When a sequence block does occur in its own upstream and downstream subsets, it indicates that the sequence block may, but not must, occur more than once in the sequence. The presence of more than one parental strand in the original mixture can introduce additional oligos into the filtered upstream and downstream subsets that can cause a block that actually occurs only once in a sequence to appear in both the upstream and downstream subsets of its own block set. However, further analysis of the data determines the multiplicity of each block in the strand (as described below), thus resolving these uncertainties. For convenience, block sets that pertain to blocks that definitely occur only once in the sequence are listed together. For example, in Figure 10d, block set "CATG" and block set "TACCTTG" are listed together.